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13. ABSTRACT (Maximum 200 words)
To enhance survivability of military personnel with limited provisions, it may be possible to alter human gut bacterial populations to increase energy yield from plant fiber. To enable development of strategies to modify bacterial function for enhanced cellulose digestion, specific aims of the project were 1) identify organisms in the pig that respond to cellulose, 2) compare these to what has been identified from humans, then 3) organisms that are cellulolytic and native (or closely related) to the human GI tract will be targeted for further characterization for future possible enhancement of fiber digestion. During this period we used comparative 16S rRNA gene sequence terminal restriction fragment length polymorphisms to identify bacteria of the swine cecum that increase in response to a high cellulose diet. The species of bacteria will be further identified from clone libraries of the 16S rRNA bacterial genes obtained from the swine cecum samples and compared to available human bacterial sequences. Preliminary sequencing has been completed and analysis of the species composition is in progress. For the next period we will complete analysis of a second diet trial, compare sequences to the available human bacterial sequences, and initiate culturing of cellulolytic bacteria native to the gut.

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Final Progress Report

August 05, 2003 – August 31, 2004

DARPA BAA03-02 Proposal: Analysis of GI community shifts in response to dietary fiber

Contract No. DAAD19-03-1-0194; Proposal No. 45612-LS

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Abstract

The human digestive system is capable of only small amounts of cellulose fermentation in the lower intestine. Since most dietary plant fiber (largely cellulose) passes through the digestive system unaltered, little carbon or energy is recovered. We suggest that it may be possible to alter the human gut community to enhance the rate of microbial digestion of fiber, resulting in increased absorption of usable energy by humans. This would enable the survival of military and intelligence personnel in the field with little provision. In order to establish feasibility, several key features of digestive system microbiota must be better characterized. Although gastrointestinal (GI) tract bacteria have been extensively studied using culture-based techniques, recent molecular analyses have revealed that as little as 25% of gut diversity is represented by bacteria in culture. Thus, relatively little is known about community structure, the physiology of uncultured populations, and the influence of diet on species composition and function. This information is essential for developing strategies to modify community structure and function. We therefore proposed to compare the microbial community structure of the human GI to that of the pig. The pig was chosen as a model system because of the similarity of its physiology and metabolism to humans. Furthermore, although the porcine diet is omnivorous and similar to the human diet, pigs recover much more energy from microbial digestion of fiber/cellulose in the lower GI tract (ca. 17-30%). In order to identify the key fiber digesting populations of the pig, we used comparative 16S rRNA sequence analysis to resolve the community composition changes induced by a high cellulose diets. These data will be related to human microbiota. Organisms that are cellulolytic and native to the human tract (or closely related to those normally resident in humans) could then be targeted for further characterization and possible engineering to increase fiber digestion efficiency.

Summary of progress this period

The primary objective of the research conducted at the University of Washington was to characterize the complex microbial populations in the swine cecum and how this community was altered by a high fiber diet (cellulose in the form of soy hulls). The Stahl lab would identify key members of the swine cecum involved in cellulose digestion in collaboration with Cherie Zeimer under subcontract with the National Swine Research and Information Center, Swine Odor and Manure Management Research Unit, Ames, IA. Bacteria were identified using culture independent molecular methods with the eventual goal of culturing targeted organisms that increased in response to high levels of fiber in the diet. The benefits of identifying these potentially cellulolytic bacteria resident in the swine cecum by molecular detection methods include: 1) increase chances of discovery of previously unidentified novel cellulolytic bacteria, 2) identification of cellulolytic bacteria in a digestive tract similar to the human tract (omnivorous, non-ruminant but better cellulose utilization), 3) show gut community response to change in diet fiber, 4) detect bacteria for further characterization as potential probiotics or for other biotechnical advances (i.e. enzymes and pathways).

At the Swine Research and Information Center, two diet trials were run with two sets of pigs fed either a low cellulose or high (10%) cellulose diet in the form of soy hulls (Table 1), and weekly fecal and cecal (via a surgically inserted cannula) samples were taken and sent to the UW for molecular analysis. Although fecal samples were also collected, analyses initially focused on cecal samples since the cecum is the site of cellulose fermentation.

The bacterial composition profiles of the cecum contents were analyzed by amplification of the 16S rRNA gene, a phylogenetically informative gene. The variability in sequences of the bacterial 16S rRNA gene were assessed by terminal restriction fragment length polymorphism analysis (T-RFLP) after restriction endonuclease digestion that provided relative abundances of bacterial members of the cecum microbial community.

Differences between the profiles of pigs on the low cellulose and the high cellulose diets were determined at the initial start of the diet trial, midway through the trial and at the end of the trial to evaluate shifts in the relative abundance of members of this community and identify population(s) that increased in response to high cellulose. Clone libraries of the 16S rRNA genes from these communities were constructed for all of the pigs for later use if needed, but only a subset actually sequenced. The bacteria that increased in abundance on the high cellulose diet were identified as members of the *Prevotella* genus in the clone libraries by sequencing these genes and comparing the fragment patterns of clones to that of the cecum samples determined by T-RFLP.

Sequences will be compared to human gut bacterial gene sequences available and those determined by David Relman at Stanford (data sharing has been established through a letter of collaborative agreement).

Work completed to date

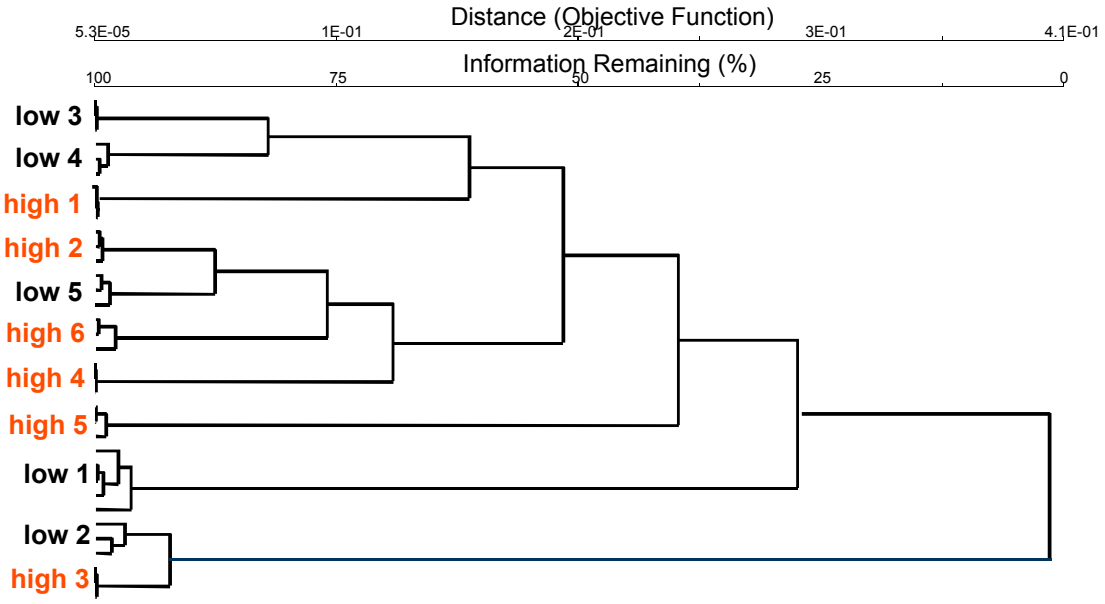
The first diet trial was conducted from mid November 2003 through mid January 2004. The second trial was conducted from about late March 2004 through late May 2004. During the set-up period for the diet trial, methods for the molecular analyses were optimized using bioreactor and fecal material provided by Cherie Zeimer. The DNA extraction and polymerase chain reaction (PCR) protocols were optimized to limit variability introduced by the methods. Based on a survey of alternative restriction enzymes, a combined digestion with two enzymes (*Hae*III and *Msp*I) was determined to provide the best resolution of resident gut microbial populations. DNA was extracted from each cecal and fecal sample of week 0, 4, 6 and 8. Amplification by PCR of the 16S rRNA gene was completed for cecal samples of both trials, and fecal samples of trial 1 only. DNA was extracted, amplification of the 16S rRNA genes completed and the terminal restriction fragment length polymorphism (T-RFLP) patterns were determined for weeks 0, 4 and 6 for each pig in each trial (11 total, 5 control and 6 high cellulose). For these analyses, triplicate PCR amplifications were run for each sample and compared. The similarity between the bacterial species composition and abundance profiles of swine samples was analyzed by cluster analysis using the statistics package PC-ORD. NMS (non-metric scaling) ordination was used to determine which members of the community (fragment) were common to cecal samples grouping together. In addition, clone libraries of the 16S rRNA gene from cecal material of all pigs were constructed. Four pigs were selected, two on the control diet and two on the high cellulose diet, for sequencing of these libraries to identify the bacterial signatures obtained by T-RFLP. Only the gene fragments from the second trial have been fully sequenced to date. Full sequences were obtained and analyzed from the libraries of pigs on low and high cellulose for week 0 and week 7 of the second trial. All libraries are available for further analysis as needed.

Summary of T-RFLP community profile analysis results

There was no statistically significant difference in the weight gain of the pigs on the two diets for either trial (data not shown). The analysis of the bacterial community TRFLP patterns showed some variance of the gut bacterial composition between pigs, similar to what has been observed in published human studies. Over the course of the diet trial the communities of both sets of pigs (control and high cellulose) shifted slightly. Cluster analysis revealed that the pigs on the cellulose diet had common peak abundance patterns indicating that a cellulose related change had occurred (Figure 1). The bacterial restriction fragments that correlated with the high cellulose diet were identified as a fragment 97 base pairs (bp) long (Figure 2). Both diet trials revealed a similar increase in the abundance of the 97 bp peak from approximately 25 % to approximately 35 % of the community when pigs were fed a high cellulose diet, despite the differences between the trial 1 and trial 2 pigs. The differences between the trial pigs included 1) starting weight, 2) gender, and 3) stress experienced from surgeries and housing. The clone library sequences showed results similar to the T-RFLP profiles in that the *Prevotella* spp. dominated both the T-RFLP profile relative abundances and the clone libraries (Figure 3). The *Prevotella* spp. were determined to increase in the high cellulose diet (peak 97 bp). In addition, members of the *Succinivibrio* genus were detected in the clone libraries but were missed in the T-RFLP profiles due to the short terminal fragment size of the digested 16S rRNA gene fragment. Certain *Prevotella* species are known to be cellulolytic. *Succinivibrio* are known starch degraders, but no info regarding their cellulolytic capabilities was found. The phylogenetic distribution of all the sequenced swine clones was consistent with what has been found previously in the mammalian digestive tract (Fig. 4). Phylogenetic analysis revealed that the *Prevotella* clones were most closely related to clones from another swine study by T. Leser [2002]. The closest relatives are shown in Fig. 5.

Initial analyses of fecal profiles revealed greater variance in the patterns between pigs than observed in the cecal material and that the composition of the cecal and fecal populations were quite distinct. These fecal profiles have not yet been rigorously analyzed since the primary focus of this study in on the cecal community, but a correlation between diet and fecal patterns community patterns was not detected.

Week 0



Week 6

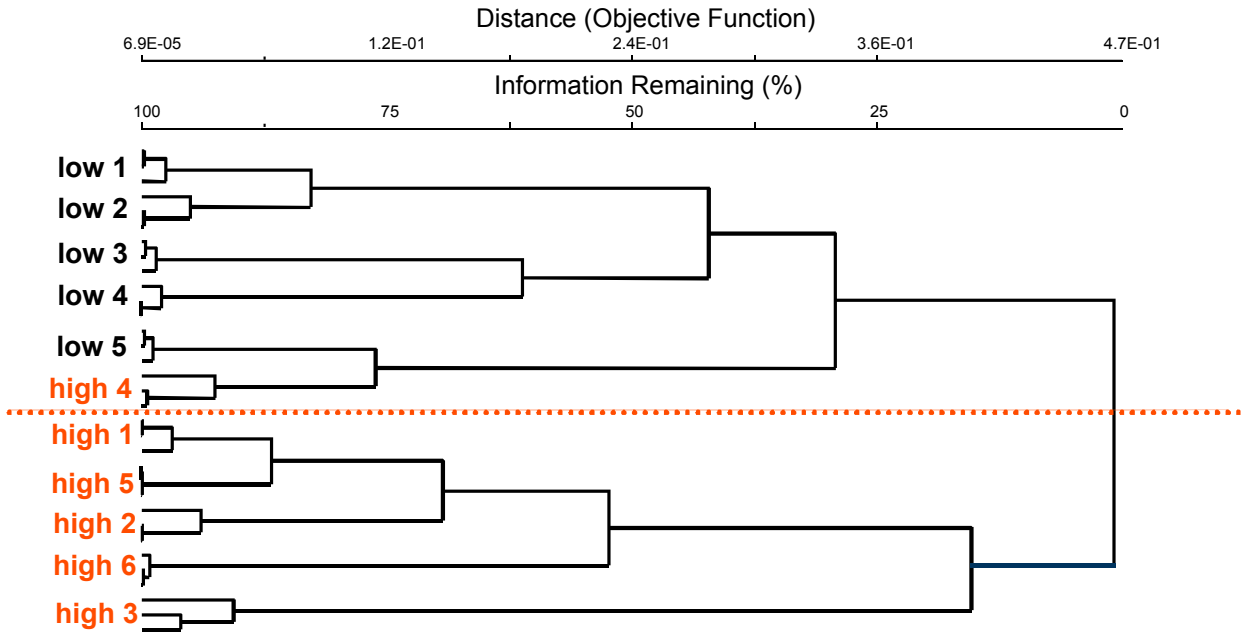


Figure 1. Cluster analysis of relative abundance patterns of bacterial 16S rRNA gene fragments from swine cecal samples over a six-week period. Analysis of both Trials 1 and 2 with all pigs combined, low=control diet and high=high cellulose diet. Branch lengths indicate relative differences between profiles of each sample.

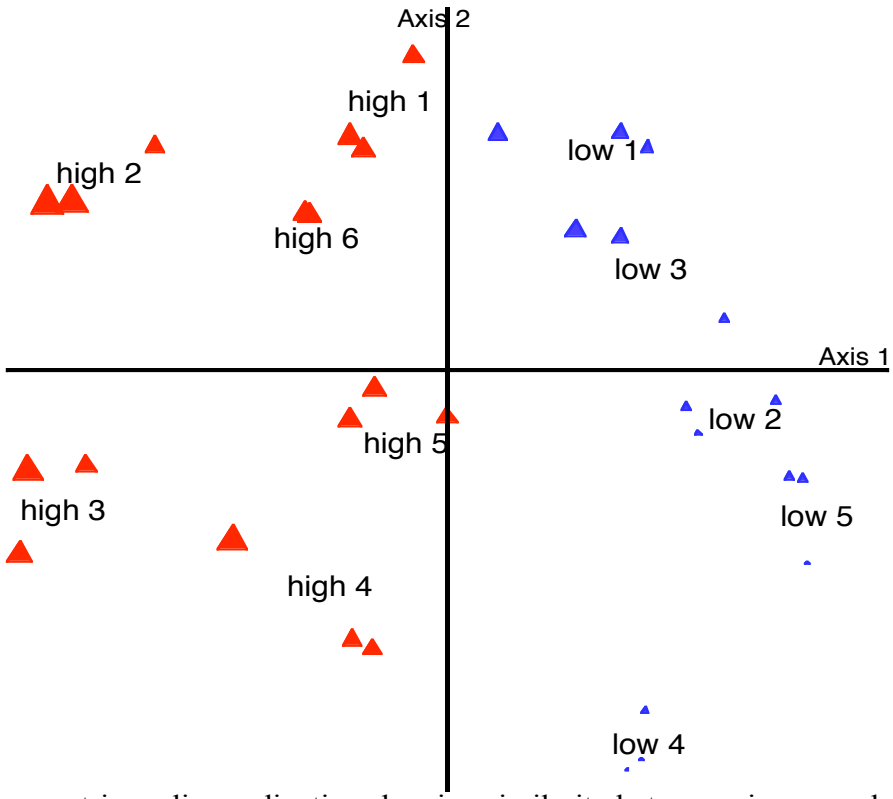


Figure 2. Non-metric scaling ordination showing similarity between pig cecum bacterial profiles at week 6 of the diet trial. Triangles illustrate relative abundance of the 97 base pair peak. The common feature that groups the high fiber cecum profiles on the left half of axis 1 is the increased abundance of fragment 97 bp. This fragment has been identified as members of the *Prevotella* genus.

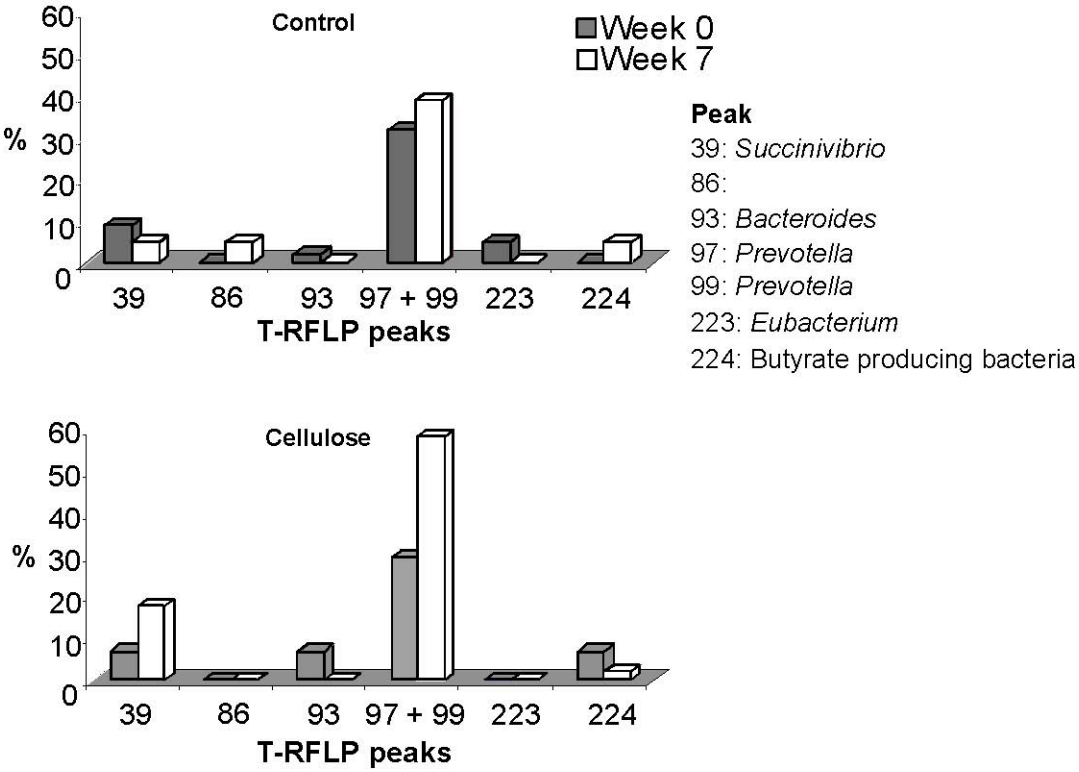


Figure 3. Composition of clone libraries from two pigs at week 0 and week 6 on the two different diets. The T-RFLP peak fragment that corresponds to the identified sequence is listed on the axis. In addition to the 97 bp peak, a few *Prevotella* clones resulted in a 99 bp fragment.

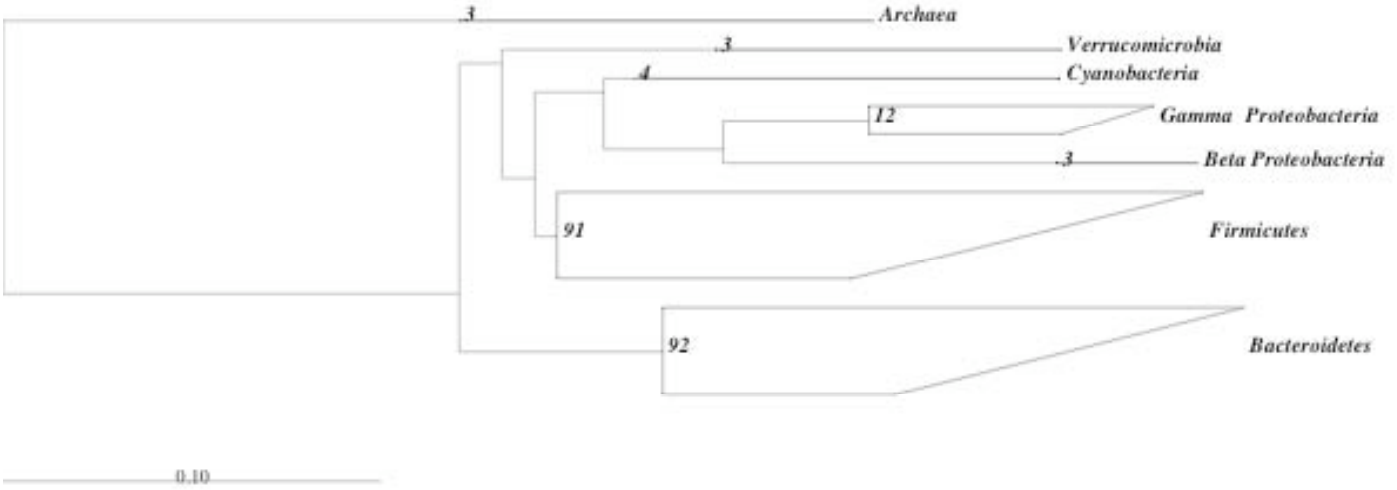


Figure 4. Phylogenetic distribution of bacteria detected in the swine cecum samples of this study.

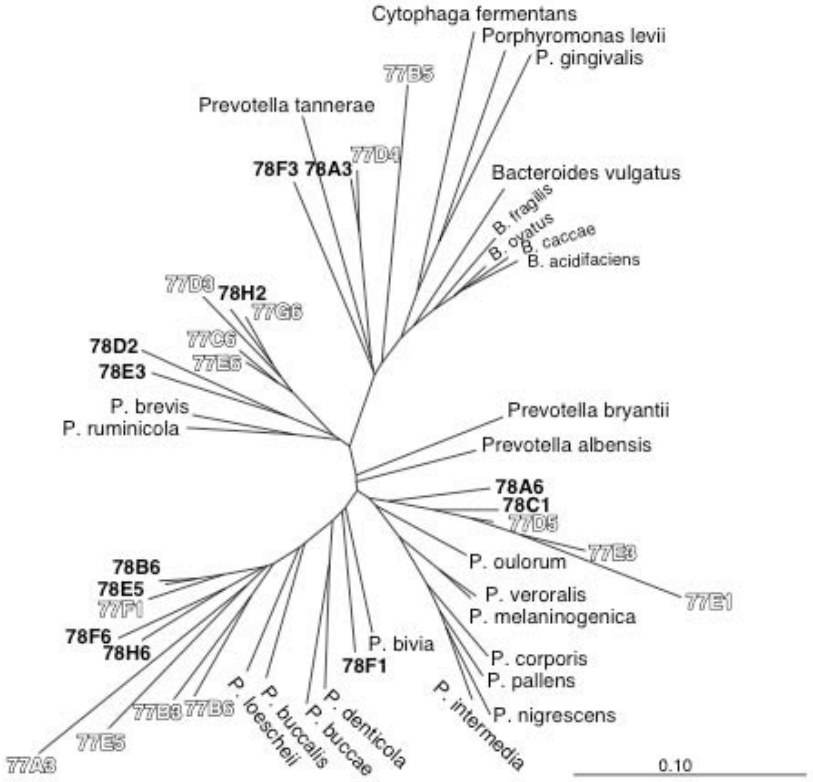


Figure 5. Phylogenetic placement of *Prevotella* clones from libraries of 16S rRNA genes amplified from cecal contents of pigs at week 7 of second diet trial. 77, low fiber diet. 78, high fiber diet. Sequences were aligned using the ARB software package (www.arb-home.de) against the 16S rRNA database with Bacteroidetes filter, and analyzed by maximum likelihood.

Table 1: Composition of Experimental Diets		
Ingredient, %	**Control**	**High Cellulose**
Corn	78.95	60.63
Soybean meal	16.31	15.36
Soybean hulls	**0.00**	**17.27**
Animal fat	2.00	4.25
Dicalcium phosphate	0.93	0.93
Limestone	0.88	0.60
Sodium chloride	0.30	0.30
ISU Vitamin mix	0.30	0.30
ISU Trace mineral mix	0.10	0.10
Choline Chloride-60	0.07	0.07
Se-270 premix	0.05	0.05
L-Lysine•HCl	0.11	0.11
L-Threonine	0.00	0.02
DL-Methionine	0.00	0.01
Calculated composition, %		
Nitrogen	2.33	2.33
Crude Protein	14.5	14.5
Neutral detergent fiber	9.08	17.69
Acid detergent fiber	2.95	10.20
Hemicellulose	6.13	7.48
Acid detergent lignin	0.28	0.85
Cellulose	**2.67**	**9.35**
All diets formulated to 3400 kcal ME/kg, .70.		